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Br. abortus Infection in Guinea Pigs. Studies in Pathogenesis and Immunity.

Ph.D. Thesis

presented by

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CONTENTS.

Part One

I N T R O D U C T I O N

A form of abortion in cattle has long been recognised as an epizootic disease, being mentioned by veterinary writers in the eighteenth and early nineteenth centuries. Its contagious nature was recognised as early as 1878 when Lehnert produced abortion in pregnant cattle by the introduction into the vagina of portions of foetal membrane from an aborting cow. The etiology of the disease was ascribed to various agents, but remained obscure until Bang, assisted by Stribolt, in 1897 obtained in culture a small Gram negative bacillus now classified as Br. abortus. They isolated the organism from the foetuses and foetal membranes of aborting cows, and established the fact that it was the cause of contagious abortion in cattle. Their work was confirmed in this country by McFadyean and Stockman (1909), in the United States by McNeal and Kerr (1910) and in Hungary by Preisz (1903).

The disease is now world wide in its distribution, and is recognised as one of the greatest scourges of the cattle breeding and dairying industries. According to Mohler (1926), Chief of the Bureau of Animal Industry, U.S.A., the economic importance of contagious abortion 'must be ranked as nearly, if not actually, supreme among the infectious diseases of domestic animals'.

Br. abortus is now also recognised as an important pathogen of man, largely through work initiated by Evans (1918). She demonstrated for the first time the close similarity between Br. abortus and Br. melitensis and the difficulty in distinguish: :ing one from the other. Previous work in London by Kennedy (1914) had shown that the serum and milk of a considerable percentage of cows contained agglutinins for the causal organism of Malta fever, and he had suggested in explanation that the agglutination was non-specific. A proper understand: :ing of this discovery was made possible by Evans' work, the agglutinins demonstrated by him being those of Br. abortus.

An important advance in the study of contagious abortion was the discovery that guinea pigs were susceptible to inoculation with the causal organism, announced simultaneously by Smith and Fabyan /

Fabyan (1912) and Schroeder and Cotton (1911). This inoculation disease had, however, already been recognised as an entity by Smith (1894) before the isolation of the causal organism by Bang and Stribolt in 1895, and McFadyean and Stockman (1909) had induced abortion in pregnant guinea pigs by inoculation with Br. abortus. Their discovery has led to the almost universal use of the guinea pig for experi: :mental studies and for the demonstration of the organism in the tissues of animals and of man.

Adequate descriptions of the inoculation disease as it occurs in guinea pigs have been recorded by several workers, of whom Fabyan (1912), Smillie (1918), Robinson (1919), Hagan (1922), Meyer, Shaw and Fleischner (1922), and Smith (1926) have studied the disease in considerable detail. All of these authors have laid emphasis on the bacteriology and particularly the histopathology of the condition but none have made a detailed study of its pathogenesis. They agreed that the inoculation of Br. abortus into guinea pigs either as a suspension of organisms grown in vitro or in milk or other cyetic products of bovines, was followed by the development of a disease characterised by enlargement of the spleen and lymph nodes and tending towards final recovery, although, in a very small percentage of cases, the animal may die from rupture of the spleen. The gross changes have been fully described by Henry, Traum and Haring (1932), while the histo-pathological aspect of the condition was first record: :ed in detail by Fabyan (1912), and later workers have done little to amplify his description.

Studies on the actual pathogenesis of Br. abortus in guinea pigs are few in number. Thus Smillie (1918) found the greatest number of organisms in the spleen 3 - 4 weeks after inoculation, but the lesions continued to increase until the 7th or 8th week, while Hagan (1922) showed that fewer than 100 organisms will infect the majority of guinea pigs and that the number of organisms recoverable declined rapidly after the 10th week.

Perusal of the available literature on the subject leaves the impression that although the pathological and histological changes in the guinea pig induced by Br. abortus have been thoroughly studied /

studied and are well known, the pathogenesis of the condition, namely the development of the bacillus in the guinea pig and the course of the infection, as distinct from the course of the disease, are but imperfectly understood.

A somewhat similar criticism may be levelled at much of the immunological work that has been done in the past with guinea pigs. Experiments with cultures suspended and killed in various ways have been recorded by Ascoli (1915), Stafseth (1920), Hagan (1922B), Gwatkin (1931) and Scales and Huddleson (1936). Living cultures attenuated in virulence were employed by Huddleson (1924), Schroeder and Cotton (1925) and McEwen and Roberts (1936). The methods of testing immunity employed by many of these authors was most probably too severe, and they concluded that killed cultures were of little value in producing immunity but that living attenuated cultures gave rather more promising results. As criteria of infection, some have merely described the size of the lesions in the immunised as compared with those in the control animals, while others have made cultures, usually from the spleen, and compared the presence or absence of infection in the two groups. None of the above workers attempted to convert the degree of infection of either their immunised or control animals to a numerical figure, with the exception of Hagan (1922) and McEwen and Roberts (1936). These authors attempted to calculate the number of organisms in the spleens of their guinea pigs by counting the number of colonies obtained on artificial media inoculated with measured quantities of tissue.

It will be shown subsequently that it is possible arbitrarily to determine numerically the degree of infection in groups of guinea pigs artificially infected with Br. abortus, and so by means of a simple calculation to determine the efficiency of different immunising agents. Should it prove possible to immunise both guinea pigs and bovines by the use of the same agent, a routine laboratory technique for the examination of vaccines used in the field has thus been developed. The possibility of immunising both species of animals by the same agent has not as yet been fully investigated, but has been more or less assumed by modern workers. The position may be summarised in the words of Huddleson /

Huddleson (1924), 'The specific agent that will protect the guinea pig should have far reaching possibilities. It should suggest the course to be pursued in the development of a preventive for the disease in the bovine'.

This thesis records the selection of a virulent strain of Br. abortus for experimental purposes, the pathogenesis of this strain and of two avirulent strains in guinea pigs, and a series of immunisation experiments in which a number of dead vaccines, four living avirulent cultures and one living virulent strain of Br. abortus were used.

Part Two

E X P E R I M E N T A L

METHODS.

General.

The experiments to be described all have as their basis the infection of groups of guinea pigs with Br. abortus, and the determination, after varying periods of time, of the degree of infection produced. This has been estimated by plating dilutions, in multiples of ten, of suspensions of body tissues on blood agar. The tissues examined in this way were the inguinal lymph node, at the site of inoculation; the submaxillary node, being that furthest from the site of inoculation; the spleen and part of the liver. The highest dilution in which Brucella was found was taken to be the degree of infection in that tissue, and was represented by the index figure of the dilution. The average of these figures for a group of guinea pigs was accepted as the degree of infection in that particular group.

The conversion of the degree of infection to a numerical figure has permitted, in the case of experiments on the production of artificial immunity, the determination of the percentage efficiency of the immunising agent in accordance with the following formula. When the perfect vaccine, that is, that which will prevent the infection of every immunised animal is represented by 100, the degree of infection in the immunised by V and in the controls by C, while the efficiency of the vaccine under discussion is E, then -

$$E = \frac{(C - V) \times 100}{C}$$

Technical Methods.

Experimental animals:

Guinea pigs of from 250 to 450 g. in weight were used throughout the experiments. Males and, so far as was possible, non-pregnant females were used in approximately equal numbers. Males, females, immunised and control animals were kept in separate cages under identical conditions. All inoculations were performed subcutaneously on the inner /

inner aspect of the hind legs. Within recent years American workers have tended to favour infection by the conjunctival route, but when dealing with an inoculum of relatively few organisms the subcutaneous would appear to be the safer method.

Media:

The medium commonly recommended for the growth of the Brucella group is liver agar (Huddleson 1939). It has the advantage that it contains no obvious pigment to affect the standardis: :ation of suspensions of organisms grown upon it. For the demonstration of Brucella in guinea pig tissues, however, sheep blood agar plates with crystal violet (B.D.H.) 1/500,000 were used. The superiority of this medium over liver agar is demonstrated in Table 1, which shows the colony counts obtained on plating 0.5 c.c. of 48 hour growths of Br. abortus standardised to No.2 on Brown's scale and on the two media.

Incubation:

Inoculated plates were incubated in an atmosphere of 10 per cent. CO_2 in metal boxes, sealed with plasticine, for four days at 37°C.

The preparation of suspensions of Br.abortus for inoculation:

The organism was grown on liver agar in tubes or flat bottles for 48 hours at 37°C. The resultant growth was washed off with normal saline and standardised to the required opacity with Brown's tubes (Gardner 1931).

The examination of guinea pigs:

All instruments, glassware, etc., were sterilised by boiling in the autoclave.

(1) The animal was killed by a sharp blow on the head, tied on a post-mortem tray and swabbed with a 2% solution of lysol. Blood was collected for an agglutination test.

(2) With the usual precautions to avoid contamin: :ation the inguinal lymph node at the site of inoculation, the submaxillary node, the spleen and part of the liver were removed to four separate weighed /

weighed deposit glasses and the weight of each determined.

(3) Each tissue was ground separately in a mortar without sand and sufficient normal saline added to make a 10% suspension. Tenfold dilutions of the suspensions were then made, using one syringe per tissue.

(4) Blood agar plates were inoculated with 1.0 c.c. of each dilution, incubated for four days and examined for the presence or absence of Brucella.

Agglutination tests:

A test was performed on the blood of every guinea pig at the time of death, and in certain cases, during the course of the infection. Blood samples for the latter were obtained by cardiac puncture. The antigen used was the standard abortus suspension prepared for the Agricultural Research Council (Stableforth 1936) and the tests were read after overnight incubation at 37°C.

TABLE 1.

The Comparison of Liver Agar and Blood Agar for the Growth of Br. abortus.

	Blood agar			Liver agar		
	10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}
1	737	26	4	680	20	4
2	1044	163	16	1000	142	13
3	746	48	7	674	27	2
4	684	84	5	410	30	2
5	780	59	6	449	54	2
6	542	90	8	389	55	3
7	566	84	10	553	39	6
8	520	73	9	536	85	8
9	582	65	4	618	54	2
10	907	114	12	735	21	6
11	875	104	16	766	89	10
Total	7983	910	97	6810	616	58
Average	725	82	8	619	56	5

THE SELECTION OF A VIRULENT STRAIN OF Br. abortus FOR EXPERIMENTAL PURPOSES.

It was apparent that before experiments on the pathogenesis of Br. abortus or on immunity could be undertaken, it was necessary to obtain a known virulent strain of the organism and to determine its minimal infecting dose for guinea pigs. Experiments were accordingly devised to obtain such a strain.

Seven cultures of Br. abortus were available for test. Their designation and origin was as follows:-

Br. abortus 'B$\frac{1}{3}$'. An old laboratory strain whose origin was unknown.

Br. abortus 'SP', 'MG', 'MGI' and 'MGII' isolated from the foetal membranes of aborting cattle.

Br. abortus 'McK'. Isolated from the vaginal discharge of an aborting cow.

Br. abortus 'G'. Isolated from the stomach contents of an aborted bovine foetus.

Groups of ten guinea pigs were infected with each strain and killed six weeks later for autopsy. The infecting dose was similar in each case and consisted of approximately 80 organisms, being 0.5 c.c. of a suspension standardised to No. 2 on Brown's Scale and diluted 10^{-7}. Both the infecting dose of organisms and the interval between infection and death were arbitrarily chosen as definite information on these points was unobtainable. Each week after infection the animals were weighed and a blood sample taken for an agglutination test.

At the time that this preliminary work was undertaken a method for the assessment of the degree of infection of groups of guinea pigs had not been developed, so a comparison of the following data was made in an attempt to determine the virulence of the cultures:- The average gain in weight of each group, the average interval between infection and the development of agglutinins, the average agglutination titre at the time of death, the number of animals infected in each group and the average weight of the spleen.

The result obtained is presented in Table /

Table 2 in which it is shown that the 'B½' and 'McK' strains are similar in being apparently the most virulent of those examined. Br. abortus 'McK' was, however, finally chosen as a virulent strain suitable for experimental work in that it produced the greater degree of splenic enlargement.

The determination of a minimal infecting dose of Br. abortus 'McK' for guinea pigs was performed in exactly the same manner.

Two groups, each of seven animals were infected with 0.5 c.c. of the 'McK' strain standardis: :ed to No. 2 on Brown's scale and diluted 10^{-7} and 10^{-8} respectively. Similar data to those recorded above were obtained and are shown in Table 3.

Although each animal in both groups became infected, the condition was somewhat more severe in those inoculated with the 10^{-7} dilution and in view of the very small number of organisms in a similar suspension diluted 10^{-8}, it was thought advisable to consider the 10^{-7} dilution an arbitrary 100% minimal infecting dose.

TABLE 2.

The Selection of a Virulent Strain of Br. abortus

Strain.	Average gain in weight.	Interval between infection and development of agglutinins.	Average agglutination titre.	Number infected in each group.	Average weight of spleen.
$B\frac{1}{3}$	152 g.	3.3 weeks	1/26,000	10	2.9 g.
SP	218 g.	5.3 "	1/1,000	7	2.0 g.
McK	174 g.	4.0 "	1/29,000	10	3.6 g.
G	238 g.	3.9 "	1/20,000	9	1.7 g.
MG	176 g.	4.7 "	1/2,000	8	1.4 g.
MGI	192 g.	4.0 "	1/500	6	1.5 g.
MGII	250 g.	3.6 "	1/9,000	8	1.9 g.
Order of Virulence					
1	$B\frac{1}{3}$	$B\frac{1}{3}$	McK	(McK)	McK
2	McK	MGII	$B\frac{1}{3}$	($B\frac{1}{3}$)	$B\frac{1}{3}$
3	MG	G	G	G	SP
4	MGI	(McK)	MGII	(MG	MGII
5	SP	(MGI)	MG	(MGII)	G
6	G	MG	SP	SP	MGI
7	MGII	SP	MGI	MGI	MG

TABLE 3.

The Determination of a Minimal Infecting Dose of Br. abortus.

Dilution of No.2 on Brown's Scale	Average gain in weight	Interval between infection and development of agglutinins	Average agglutination titre	Number infected in each group	Average weight of spleen
10^{-7}	163 g.	2.2 weeks	1/23,000	7	3.7
10^{-8}	195 g.	4.0 weeks	1/19,000	7	2.5

EXPERIMENTS ON THE PATHOGENICITY OF Br. abortus.

The pathogenesis, namely the course of the infection, of three strains of Br. abortus was determined in guinea pigs. One virulent and two avirulent strains were used.

The virulent strain, Br. abortus 'McK' had been subcultivated approximately thirty times since its original isolation.

The avirulent strains were, one, Br. abortus '45, guinea pig passage generation 7' received from Dr McEwen of Wye, Kent, and two, Br. abortus '805', an American strain received from Dr Huddleson of Michigan.

The Pathogenesis of Br. abortus 'McK'

Each of 300 guinea pigs received a minimal infecting dose of the McK strain, approximately 80 organisms, namely 0.5 c.c. of a suspension standard:ised to No.2 on Brown's scale and diluted 10^{-7}, subcutaneously inside the right hind leg. On the day following inoculation, and every day thereafter for a period of 14 weeks three guinea pigs were killed and the degree of infection of each determined.

The result obtained is shown in Table 4 in which each figure represents the average of twenty-one tissues, and graphically in figure I.

During the first week after inoculation the organism was recovered from the inguinal lymph node only, but by the second week it had become more widely distributed and was isolated from the submaxillary lymph node and from the spleen. Dissemination throughout the body, as indicated by constant infection of the liver did not occur until the third week after inoculation, by which time the infection was well established in the other tissues examined. The predilection of Br. abortus for lymphoid tissue, which in the guinea pig becomes greatly enlarged with infection, was noted by the early workers, while more recently Doyle (1935) in an investigation into the distribution of Brucella in the system of carrier cows, was able to isolate the organism from 13 different sites, 8 of which were lymphoid tissue.

The degree of infection continued to increase and /

and reached its maximum during the fifth week after inoculation. No appreciable decrease occurred until the seventh or eighth week, when it gradually declined until the fourteenth week, when the experiment terminated. It will be shown later that it would have been necessary to run the experiment for a further eight or nine months to follow the infection to its conclusion, i.e. when Brucella could no longer be isolated from the guinea pigs.

These findings are not in close agreement with those of Smillie (1918) and Smith (1926). The former found that the greatest number of organisms was present during the 3rd and 4th weeks after infection, when it declined, while Smith also found the bacilli scarce in the tissues after the 8th week. The discrepancy between their findings and those recorded above is explained in part by the work of Hagan (1922 A), who although describing a rapid fall in the number of organisms recoverable after the 10th week, showed that the disease in guinea pigs may be prolonged by the use of a small inoculum.

A positive agglutination titre (see figure I) was not obtained before the third week after inocula: :tion. It then rose gradually until after the seventh or eighth week, when the infection was at its height, and may not have reached its peak when the experiment terminated. This is of some practical interest as it emphasises the difficulties encountered in attempting to eradicate Brucellosis from cattle by means of the agglutination test, and points to the need for frequent testing, especially of animals which react in a low titre. Thus the infection may be actively progressive yet the agglutination titre remains negative or positive in only low dilutions, or the infection may be retrogressive and the agglutination titre strongly positive. This point will be demon: :strated further in one of the experiments on immunity to be described.

The Pathogenesis of Br. abortus 'McE G.P.P.G.7' and Br. abortus '805'

Experiments on the pathogenesis of the two avirulent strains were conducted in order to obtain a basis of comparison between them and the virulent 'McK' strain and to determine if possible the length of time that they persisted in the guinea pig.

It was believed (McEwen and Roberts 1936; Huddleson, private communication) that the 'McE' strain persisted /

persisted in the guinea pig for approximately five weeks, and strain '805' for three weeks. Accordingly two groups of guinea pigs were inoculated subcutaneous: :ly with strains 'McE' and '805' respectively, the dose of each being 2.0 c.c. of a suspension standar: :dised to No.9 on Brown's scale, approximately 14,000 million organisms. On the day following inoculation, and every day thereafter, three guinea pigs of each group were destroyed and the degree of infection determined as before. Unfortunately, both strains persisted in the guinea pig for a longer period than had been anticipated, and despite a severe curtail: ment in the number of animals examined daily, it was not possible to continue the experiments to their logical conclusion, i.e. when the infection became completely negative.

The results obtained are shown in Table 4 and graphically in figure II. The degree of infection is that of the guinea pigs as a whole, and not of each individual tissue. The pathogenesis of the 'McK' strain is included in similar form for comparison.

The degree of infection of both groups of guinea pigs fell rapidly after inoculation until by the fourth week the '805' strain had been almost completely eliminated, while the 'McE' strain persisted for, presumably, rather more than seven weeks.

Although the experiments must be regarded as incomplete, both the avirulent strains, while apparently incapable of establishing themselves in the guinea pig, are able to multiply to some extent, as evidenced by the time necessary for their elimin: :ation.

In both groups of animals specific agglutinins were demonstrable within one week of inoculation. This rapid appearance was in all probability due to the relatively enormous infecting dose involving the immediate flooding of the animal body with organisms and the consequent shortening of the lag phase. The agglutination titre in each case rose almost imme: :diately to its peak and then fell with the elimin: :ation of the bacilli. This is in marked contrast to the behaviour of the agglutination curve stimulated by the 'McK' strain, which continued to rise after the infection had passed its maximum. The true explanation of this phenomenon is difficult to understand, and remains obscure.

TABLE 4.

The Pathogenesis of Br. abortus 'McK'

Weeks after Inoculation	Degree of Infection.			
	Inguinal lymph node	Submaxillary lymph node	Liver	Spleen
1	1.0	0	0	0
2	3.6	0.2	0	0.3
3	5.3	2.2	1.2	3.0
4	5.3	3.6	2.3	4.5
5	7.0	4.9	3.3	6.4
6	6.8	5.3	2.2	5.5
7	7.7	6.2	3.1	6.8
8	7.2	5.8	2.5	4.9
9	5.5	4.7	2.8	5.3
10	5.8	4.9	2.9	5.4
11	5.5	4.7	2.3	4.0
12	4.5	3.5	1.8	4.3
13	4.8	4.4	2.3	4.8
14	4.5	4.0	2.2	4.5

TABLE 5.

The Pathogenesis of Three Strains of Br. abortus.

Virulent Strain 'McK'			Avirulent Strain 'McE G.P.P.G.7'			Avirulent Strain '805'		
Time	Degree of Infection	No. of Guinea Pigs	Time	Degree of Infection	No. of Guinea Pigs	Time	Degree of Infection	No. of Guinea Pigs
1st week	0.25	21	1st week	3.6	21	1st week	3.4	21
2nd "	1.1	"	2nd "	2.2	"	2nd "	1.6	21
3rd "	2.9	"	3rd "	1.8	"	3rd "	0.9	19
4th "	4.0	"	4th "	2.1	"	4th "	0.08	14
5th "	5.4	"	5th "	1.6	16	5th "	0.03	8
6th "	5.1	"	6th "	0.6	7			
7th "	6.0	"	7th "	1.1	7			
8th "	5.1	"						
9th "	4.6	"						
10th "	4.8	"						
11th "	4.2	"						
12th "	3.6	"						
13th "	4.1	"						
14th "	4.0	"						

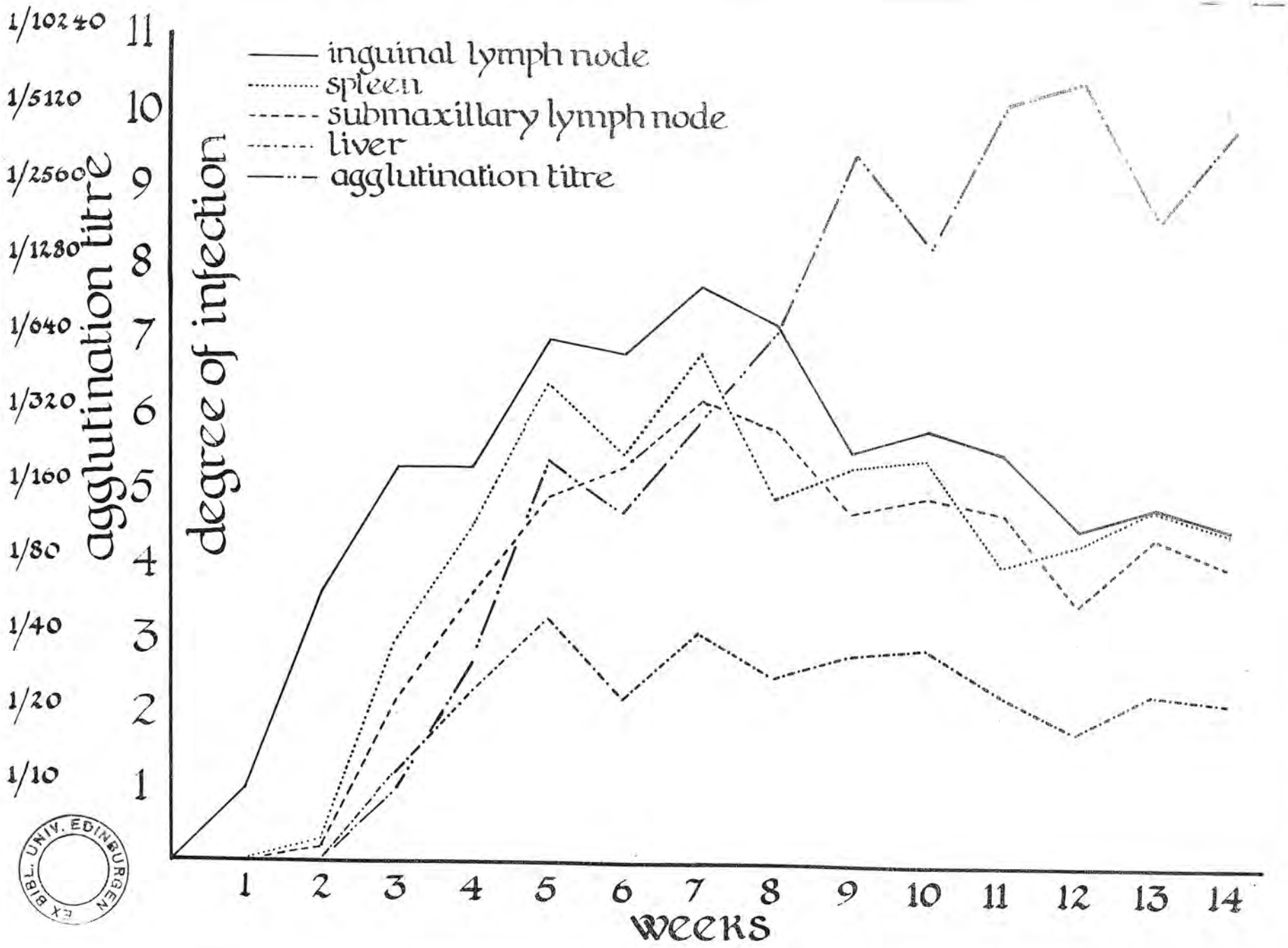

Figure I.
Pathogenesis of Br. abortus 'McK'.

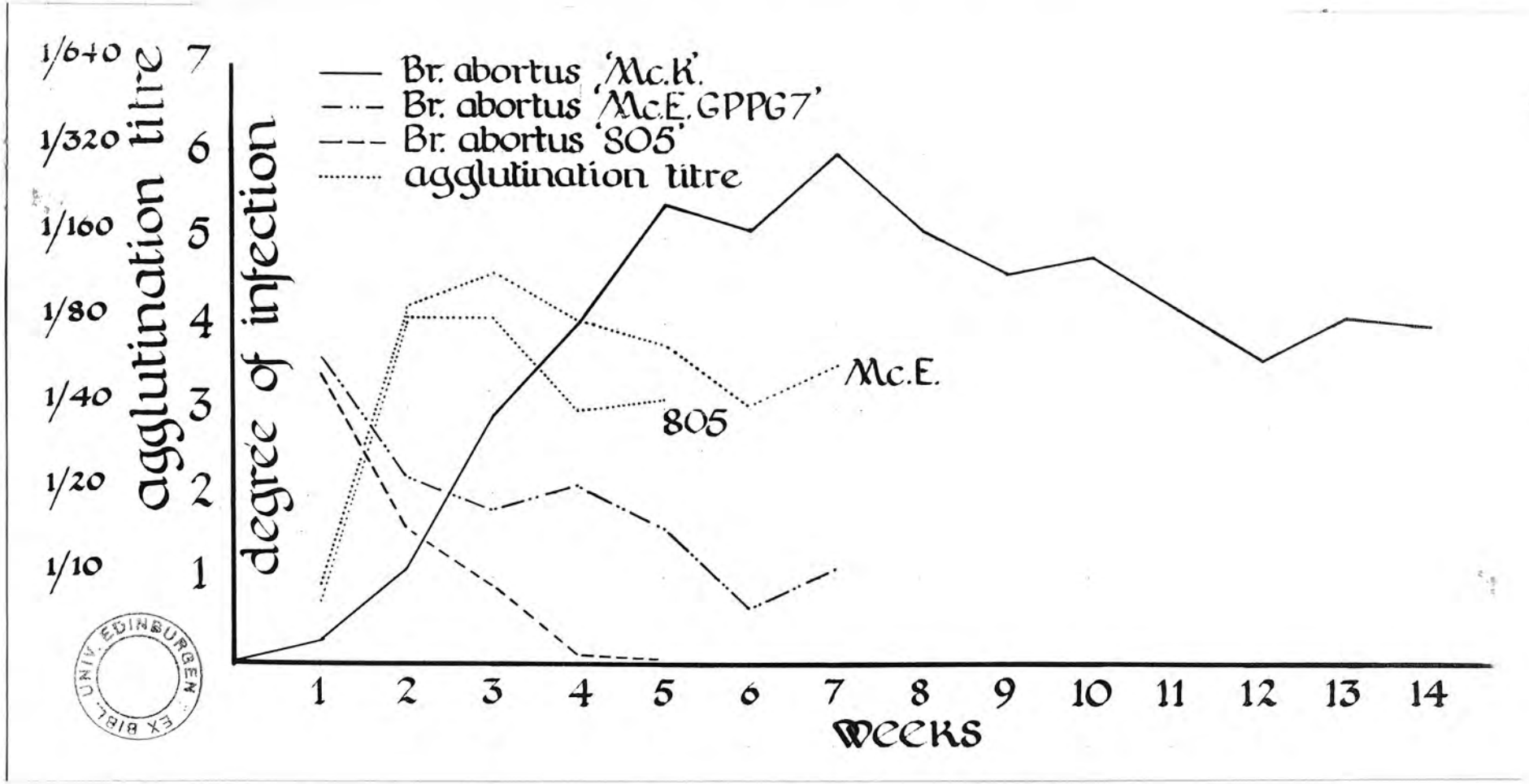

Figure II.

Pathogenesis of Three Strains of Br. abortus.

EXPERIMENTS ON IMMUNITY.

Apart from the vaccines or other immunising agents to be used, there are certain essentials to be acquired before a series of experiments on the production of artificial immunity in guinea pigs can be undertaken. It is necessary, firstly, to have a virulent strain of Br. abortus for the purpose of testing the immunity that may be engendered in the vaccinated animals, and to know the minimal infecting dose of that strain for guinea pigs. Secondly, a knowledge of the time after infection that the disease is at its height, and thirdly, a method of comparison of the degree of infection in immunised and control animals are required. This information was available as a result of work already described. A suitable infecting dose of virulent Br. abortus was 0.5 c.c. of a suspension of the 'McK' strain standardised to No.2 on Brown's scale, diluted 10^{-7}, and inoculated subcutaneously. The inoculation disease has been shown to reach its height between the fifth and eighth week, while a method of assessing the degree of infection of groups of guinea pigs has been described.

Two types of immunising agent were used, (1) dead vaccines, i.e. suspensions of killed organisms, and (2) living cultures of which four were avirulent for the guinea pig and one was a virulent strain of Br. abortus.

Dead vaccines:

(1) Formalinised guinea pig spleen.

The spleens of ninety-five guinea pigs, which six weeks previously had been heavily infected with Br. abortus (McK strain), were ground in a mortar with normal saline to make a 20% suspension of spleen. The mixture was thoroughly shaken in a mechanical shaker, filtered through butter muslin to remove gross particles and divided into two lots. A potency test on blood agar demonstrated the presence of Brucella in considerable quantity. Formalin (40% formaldehyde) was added to one lot of suspension to make a concentration of 0.1%, which was then incubated for 24 hours. The material was tested for sterility and stored in the cold without further preservative.

(2) Heated guinea pig spleen.

The second quantity of suspension described above was placed in a water bath at room temperature and the temperature raised to 60°C., which was

maintained /

maintained for 10 minutes. The vaccine was found to be sterile and was stored as before.

(3) Formalinised culture of Br. abortus.

The 'McK' strain of Br. abortus was grown on liver agar for four days at 37°C. The resultant growth was examined for purity, standardised to No.9 on Brown's scale (7,000 million organisms per c.c.) and divided into two lots. To one formalin was added to make 0.1 per cent. The vaccine was incubated for 24 hours, tested for sterility, and stored in the cold without further preservative.

(4) Heated culture of Br. abortus.

The second quantity of culture described above was heated, tested and stored in a similar manner to No.2.

(5) Formalinised 1st. subculture from guinea pig spleen.

The spleens of eight guinea pigs, heavily infected with Br. abortus, McK. strain, six weeks previously, were ground in a mortar with normal saline to make a 20 per cent. suspension of spleen. This material was sown on liver agar and incubated for four days in 10 per cent. CO_2 at 37°C. The resultant growth was examined for purity and standardised in normal saline to No.9 on Brown's scale. Formalin was added to half of the suspension to make 0.1 per cent. which was then incubated, tested and stored as before.

(6) Heated 1st. subculture from guinea pig spleen.

The second quantity of culture described above was heated, tested and stored in a similar manner to No.2.

(7) Formalinised foetal stomach contents.

The stomach contents of a bovine foetus aborted at 7 months were removed aseptically with a pipette. A small quantity, when plated on blood agar and incubated for four days in 10 per cent. CO_2 gave a profuse growth of Br. abortus in pure culture. The remaining contents were mixed with sterile broth in the proportion of one volume of contents to two volumes of broth, thoroughly shaken and filtered through butter muslin to remove gross particles. Formalin was added to make 0.25 per cent.; the mixture was incubated for twenty-four hours, tested for sterility and stored in the /

the cold without additional preservative.

(8) Formalinised 1st. subculture from foetal stomach contents.

The stomach contents of an aborted bovine foetus were removed aseptically, diluted one volume in four with sterile broth and thoroughly shaken. The mixture was then sown on liver agar and incubated in 10 per cent. CO_2 for five days. The resultant growth was examined for purity and standardised in normal saline to No.9 on Brown's scale. Formalin was added to make 0.1 per cent. prior to incubation for twenty-four hours. Sterility tests were performed and the vaccine stored in the cold without further preservative.

(9) Formalinised broth culture.

500 c.c. of broth were inoculated with a recently isolated strain of Br. abortus and incubated for ten days at 37°C. The culture was examined for purity, formalin added to make 0.1 per cent. and the whole incubated a further twenty-four hours. Sterility tests were performed and the vaccine stored in the cold as before.

(10) & (11) The Ministry of Agriculture (Northern Ireland) Br. abortus vaccine.

This is a very heavy suspension of Br. abortus whose preparation has been described by Kerr, Lamont and Shanks (1935). The author is indebted to Mr Lamont, Head of the Research Division, Stormont, Belfast, for supplies of this product.

The remaining dead vaccines examined were commercial products marketed by the various manufac: :turers concerned.

(12) & (13) 'Amblosin' vaccine. (Messrs Bayer Products Ltd., London).

(14) 'Yatren' vaccine (Messrs Bayer Products Ltd., London).

(15) & (16) 'Abortus chinosol' (International Serum Co., Norwich).

(17) 'Nator B.' (Messrs Genatosan Ltd., London).

(18) 'Rakulin' (Messrs C. Zimmermann & Co., London).

Living Cultures.

Living avirulent vaccines:

(19) & (20) Br. abortus '45, guinea pig passage generation /

generation 7', received from Dr A.D. McEwen of Wye, Kent.

(21) Br. abortus '45, generation x 24', also received from Dr McEwen.

(22) Br. abortus '805' received from Dr I.F. Huddleson of Michigan, U.S.A.

(23) Br. abortus '85X' also sent by Dr Huddleson. This strain was incapable of stimulating the production of specific agglutinins, and also differed in chemical composition from virulent strains of Br. abortus (Hershey & Huddleson 1936).

Living virulent vaccine:

(24) The 'McK' strain of Br. abortus was used.

Vaccination.

Dead Vaccines:

Five weekly doses of 2.0 c.c. of vaccine were given subcutaneously on the inner aspect of the hind leg to each animal, making a total of 10.0 c.c. per guinea pig. In the case of the commercial vaccines, the same procedure was followed, as the manufacturers' instructions in regard to dosage, etc., could obviously not be performed in laboratory animals.

Living Avirulent Vaccines:

A single dose of 2.0 c.c. of a four day culture on liver agar, standardised in normal saline to No. 9 on Brown's tubes, approximately 14,000 million organisms, was given subcutaneously.

Living Virulent Vaccine:

This experiment will be described separately.

Infection.

A minimal infecting dose of Br. abortus, 'McK' strain, was used. A larger test dose was not employed as it might have overwhelmed any immunity that might have been produced.

The vaccinated animals, with a number of controls /

controls, were given a standard test dose of Br.abortus subcutaneously in the right hind leg two weeks after the last injection of dead vaccine, and in the case of the living avirulent vaccines, six weeks after inoculation. The greater interval between vaccination with living organisms and infection was observed in order to permit of the former being eliminated from the animal body. The actual dosage varied somewhat as follows:- The groups of guinea pigs vaccinated with Nos. 1 to 6 inclusive, and Nos. 12, 14 and 15 received 0.5 c.c. of Br. abortus 'McK', standardised to No. 2 on Brown's scale and diluted 10^{-7} in normal saline. The remainder received a similar dose but diluted 10^{-6} in normal saline. This increase in the number of organisms used to test immunity became necessary because of an apparent decrease in the virulence of the 'McK' strain. The guinea pigs immunised with Nos. 19 and 20 received a test dose of organisms diluted 10^{-7} in the usual manner, but it appeared from agglutination tests that in all probability the control animals were not infected, so a second test dose, diluted 10^{-6} was given, fourteen days after the first to No. 19 and seven days later to No. 20. Direct counts were made on blood agar plates, incubated for seven days in 10 per cent. CO_2, of the '10^{-6}' test doses inoculated, and the average number of colonies counted was 884. The same dilution of a culture of Br. melitensis should contain 800 organisms (Gardner 1930). (It should be noted that the vaccines were not tested in numerical order, but are arranged thus for convenience of description).

Examination of Guinea Pigs.

The guinea pigs, both immunised and controls, were killed six weeks after infection with the test dose and the degree of infection of each group calculated as before.

Results.

The results obtained are shown in Table 6 and graphically in figures III and IV.

Vaccine No.24. Living Virulent Culture

It was hoped, by means of this experiment, definitely to determine whether it was possible to immunise guinea pigs effectively against infection with Br. abortus. Accordingly, it was arranged that a number of guinea pigs be inoculated with a suspension of living virulent Br. abortus and the subsequent infection of each animal demonstrated

by /

by the agglutination test. The tests would be continued until the average agglutination titre had reached its maximum and had begun to fall, when the presence or absence of infection would be determined. If the animals were no longer infected, a number, along with controls, would receive a test dose of Br. abortus and be examined in six weeks. The remain: :der would be retained until the average agglutination titre became negative or remained stationary, when they also would be re-infected and examined after a further six weeks. Unfortunately, the latter part of the experiment, i.e. the determination of the immunity of animals whose agglutination titre had become negative, was not carried through, as all the remaining immunised animals died of epizootic disease.

The rest of the experiment was, however, performed as follows:-

249 guinea pigs received a subcutaneous inoculation of 0.5 c.c. of a suspension of Br. abortus, 'McK' strain, standardised in normal saline to No.2 on Brown's scale and diluted 10^{-4}. A direct count on blood agar plates of higher dilutions showed that approximately 110,000 organisms were inoculated into each guinea pig.

Twelve weeks after vaccination each animal was bled and the average agglutination titre determined. This procedure was repeated every four weeks until thirty-two weeks after vaccination, as shown in Table 7.

Thirty weeks after vaccination, five guinea pigs were killed and blood agar inoculated with the spleens of each. No Brucella was isolated, so two weeks later, twenty animals were killed and examined in a similar fashion. Again, Brucella was not isolated, except from the spleens of two of these animals, each of which yielded a very few colonies. It was assumed, however, that generally speaking, the guinea pigs were no longer infected.

Consequently, thirty-three weeks after vaccination, fifty vaccinated animals and fifty normal control animals of similar size each received a test dose of Br. abortus, being 0.5 c.c. of a suspension of the 'McK' strain, standardised in normal saline to No. 2 on Brown's scale and diluted 10^{-4}. Direct counts of higher dilutions on blood agar showed that each animal received approximately 120,000 organisms.

Six weeks later, both immunised and control animals were killed and the degree of infection in each /

each group determined, with the result shown in Table 6 and figure IV. In this instance the degree of infection was calculated from the spleen only, as the other tissues, i.e., inguinal and submaxillary lymph nodes, and the liver, were not cultured as they had been in all previous experiments on immunity. They were omitted in this case partly to effect a considerable saving in time and material, and partly because it was thought that the degree of infection in the spleen gave an adequate representa: :tion of the infection in the animal as a whole.

The average agglutination titre of the remaining immunised animals was determined every four weeks until fifty-six weeks after vaccination, by which time it had fallen to 1/47. At this stage, as previously described, all the guinea pigs died during the course of an epizootic, thus terminating the experiment.

TABLE 6.

Vaccine Number	Number of Vaccinated Animals	Number Infected	Number of Control Animals	Number Infected	Degree of Infection		% Efficiency
					Vaccinated	Controls	
1	7	7	7	7	4.6	5.2	11
2	12	12	7	7	4.4	4.7	6
3	8	7	7	7	4.0	4.9	18
4	11	8	7	7	3.7	4.0	7
5	11	11	7	7	3.9	5.0	22
6	13	13	7	7	3.8	4.9	22
7	15	15	7	7	4.1	5.0	18
8	8	8	8	8	4.2	5.1	17
9	13	13	3	3	4.5	5.4	16
10	19	13	7	7	2.8	4.9	42
11	32	22	25	25	3.0	3.9	23
12	20	7	6	4	1.1	3.8	71
13	45	37	23	23	3.1	4.2	26
14	20	10	8	6	2.4	4.2	42
15	18	10	8	8	2.8	5.9	52
16	30	29	24	24	2.2	2.0	Negative
17	18	18	8	8	4.2	4.0	Negative
18	19	19	8	8	4.0	4.2	4
19	20	7	8	8	1.1	5.2	79
20	44	19	25	25	0.8	3.6	77
21	20	14	8	8	2.8	4.8	41
22	28	24	18	18	3.6	4.7	23
23	14	13	8	8	4.7	4.2	Negative
24	44	4	45	43	0.2	4.0	95

TABLE 7

Weeks after Vaccination	Number of Guinea Pigs	Average Agglutination Titre
12	197	1/1300
16	194	1/1132
20	190	1/1030
24	174	1/393
28	164	1/480*
32	146	1/144

* This figure was recorded by an assistant in the absence of the author.

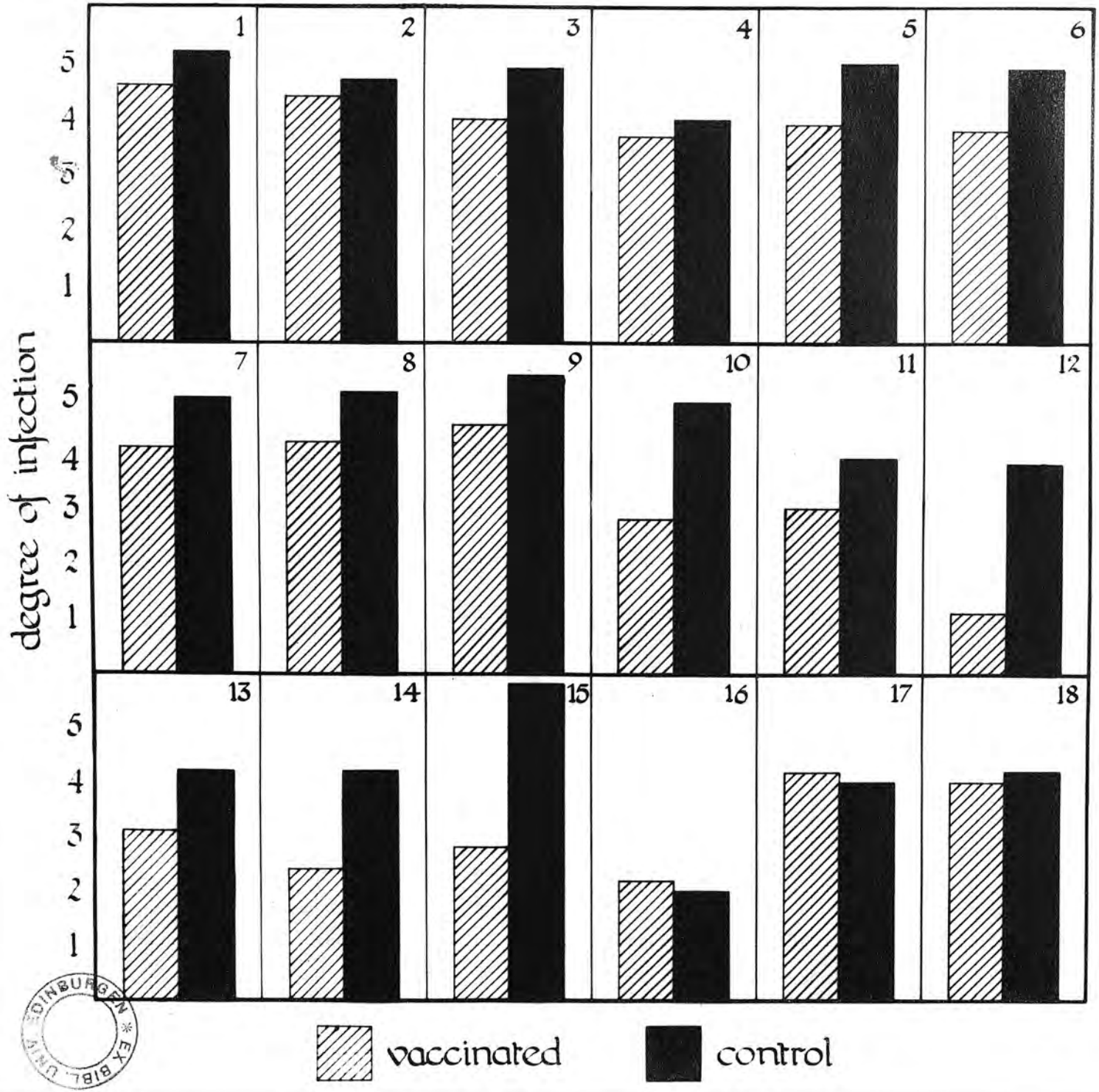

Figure III.

Immunity Experiments. Dead Vaccines.

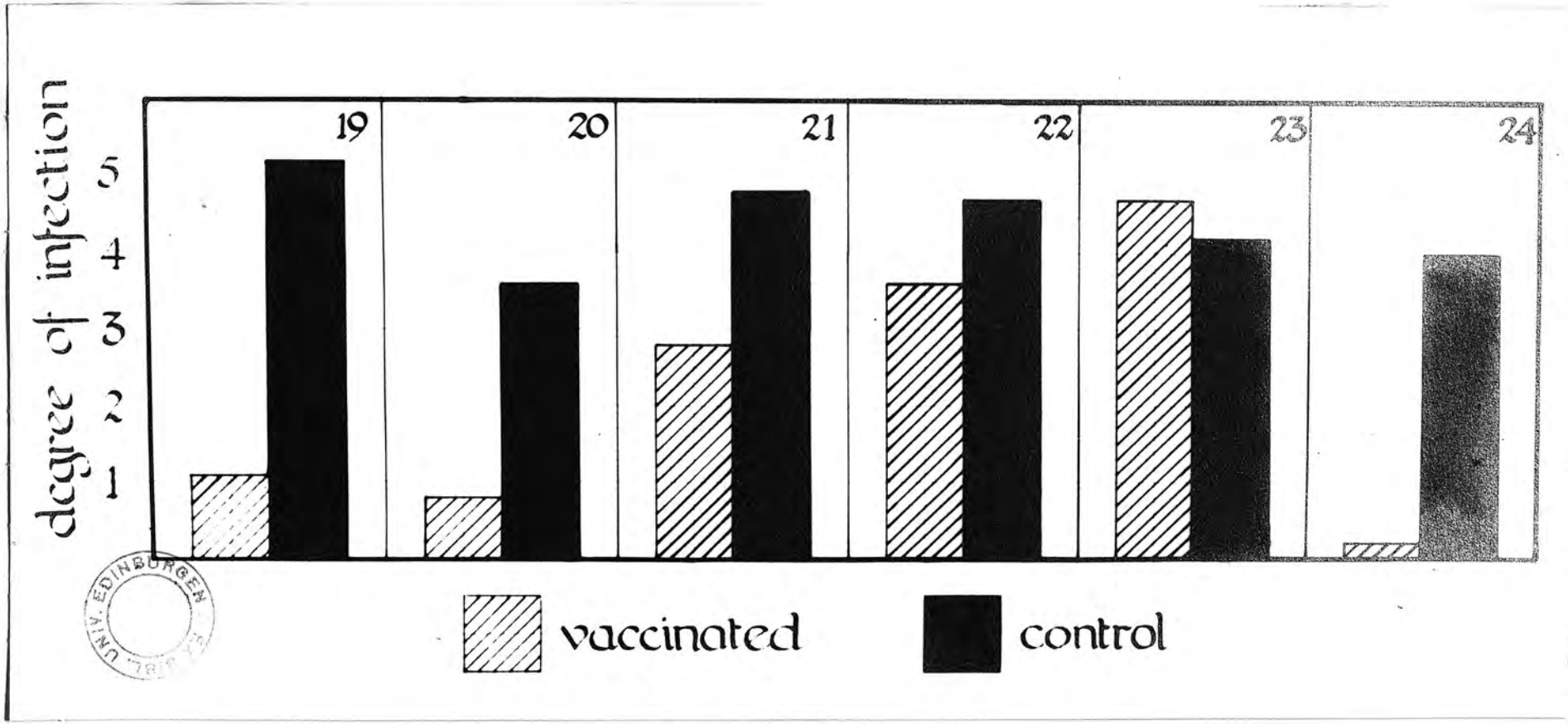

Figure IV.

Immunity Experiments. Living Vaccines.

Part Three

D I S C U S S I O N

DISCUSSION.

The great economic importance of Brucellosis in cattle, not only in this country, but throughout the world, and the intimate association between the disease in animals and in man, has given rise to a relatively enormous literature. Every aspect of the disease and its causal organisms have been closely studied over the last forty years, but from the veterinary standpoint at least, the greatest effort has been directed towards immunisation. Numerous workers in this field have conducted experiments both in the natural host and in laboratory animals, and of these, the guinea pig has proved the most suitable and has been most commonly used. Despite the volume of published work, however, an exact knowledge of the pathogenesis of the 'inoculation disease' as it occurs in the guinea pig was unavailable, and no standard method for the evaluation of immunising agents in laboratory animals had been evolved.

The main object of the work which forms the basis of this thesis has been to find, if possible, a method for the immunisation of guinea pigs against infection with Br. abortus.

Prior to undertaking such research, however, it was considered necessary to obtain a fundamental knowledge of the disease in the guinea pig. With this end in view the pathogenesis of one virulent and two avirulent strains of Br. abortus was studied in some detail. The virulent strain, inoculated as a minimal infecting dose, multiplied in the guinea pig until the infection reached its height between the fifth and eighth weeks. Thereafter it gradually decreased, and as a later experiment showed (Vaccine 24) is finally eliminated eight or nine months after inoculation. Neither of the avirulent strains, inoculated in massive doses, were capable of establishing themselves in the guinea pig, and both were eliminated within two months of inoculation. It is unlikely that such strains exist in nature, but they appear to be comparatively common among the stock strains of Br. abortus maintained in laboratories, and it is conceivable that many discrepancies in the literature may be due to the use of such strains experimentally. There can be no doubt moreover, that strains of Br. abortus of all degrees of virulence for the guinea pig are obtainable, varying from the recently isolated strain capable of producing gross enlargement of the spleen and lymphatic tissues, to the old laboratory culture, incapable /

incapable of producing lesions and eliminated by the guinea pig a few weeks after inoculation.

Having obtained a more intimate knowledge of the disease in guinea pigs, it became possible to undertake a series of experiments on immunity.

It was at once apparent, that a suitable system for the comparison of immunised and control animals was essential, and the methods of the earlier workers, namely a comparison of the extent of the lesions in each group, or more rarely the attempted estimation of the numbers of organisms in the spleen, did not appear entirely satisfactory. The method employed throughout this work, however, has permitted the expression of the degree of infection of groups of animals as a numerical figure; and in consequence, a more adequate comparison of the effects of vaccination on immunity could be made. In fact, a standard method for the assessment of vaccines against Br. abortus in guinea pigs has been evolved and used throughout the experiments. The method might equally well be applied to trials with chemotherapeutic substances, although such experiments are outside the scope of the present work.

The experience gained with dead vaccines has been that of many other workers in this field, namely that guinea pigs cannot be efficiently protected against 'inoculation Brucellosis' by the injection of killed cultures. There was but little difference between the lethal effect of formalin and of heat, both not only destroyed the organism but also its major antigenic components. It is generally recognised that bacteria tend to vary antigenically on prolonged subcultivation in the laboratory, as was demonstrated by Grinnell (1932) working on the typhoid bacillus. This principle was applied to Br. abortus; both the naturally occurring organism and primary cultures being used as vaccines, but without result. Immunising agents prepared from the organs of infected animals have been used with success in the protection of animals and man against such virus diseases as rabies, dog distemper and louping-ill in sheep, but vaccines prepared in a similar fashion from tissues heavily infected with Br. abortus have proved valueless; this was also the experience of Scales and Huddleson (1936).

Tissue vaccines, suspensions of the naturally occurring organism, cultures on solid and in liquid media, all were equally inefficient in stimulating a serviceable immunity. It is commonly accepted that there is no direct correlation between agglutination /

agglutination titre and immunity, for every dead vaccine tested produced an agglutination titre in greater or lesser degree. One in particular, the 'Northern Ireland' vaccine (Nos. 10 and 11), produced in one animal an agglutination titre of 1/2560 prior to inoculation with the test dose, yet none produced a serviceable degree of immunity.

The five widely advertised commercial vaccines examined were alike in their inability to stimulate immunity in guinea pigs. It is perhaps surprising that the comment of Huddleson (1924) that 'The worthlessness of a killed vaccine in protecting the guinea pig ... ... should furnish considerable enlightenment on the same agent now being advocated for use in the bovine by some of our prominent biological manufacturers' should still require emphasis in this country eighteen years after it was written. The Bureau of Animal Industry of the United States ceased to issue licences for the manufacture of dead vaccines in 1934. (Fitch and Donham 1934).

It has long been recognised that in a herd of cattle infected with Br. abortus the great majority of cows that abort do so but once, and only a very small proportion abort three times (Bang 1897). Presumably, therefore, one attack of the disease confers a lasting immunity. This was early taken advantage of by Bang (1906) and by Stockman (1914), who reported a certain degree of success in lowering the number of abortions in cattle by the inoculation of living cultures of Br. abortus. From that date until recent years many thousands of cattle in this country have received inoculations of living Br. abortus in an effort to control the disease, but without exerting any apparent effect upon its incidence. The vaccine has commonly been used in a somewhat casual fashion, and adequately controlled large scale experiments on its use are conspicuous by their absence. Of late years it has been increasingly realised that the use of virulent culture as a vaccine tends to cause the organism to localise in the udder and to be excreted in the milk, so becoming a possible menace to the public health (Cotton, Buck and Smith, 1933). Further, whether the immunity, if any, engendered is a true systemic immunity has never been fully determined.

The experiment described with living virulent culture (No. 24) has shown, however, that in guinea pigs at least, it is definitely possible to produce a high degree of immunity to infection with Br. abortus, and further, that this immunity is not dependent upon /

upon continued infection with the specific organism.

The use of a virulent culture as a vaccine in any infectious disease, however, is to be deprecated, and the particular disadvantages inherent in its use in the case of contagious abortion have, in recent years, prompted the investigation of avirulent or attenuated strains of the organism for use as immunising agents.

The first recorded attempt at immunisation against Brucellosis by the use of living avirulent culture was that of Huddleson (1924), since when many American workers have studied its value. In this country its possibilities have been investigated mainly by McEwen and Roberts (1936) and McEwen (1937, 1938A, 1938B, 1938C). The majority of these workers report favourably on its use.

Unfortunately, but as is to be expected, all avirulent strains do not appear to be of even approximately equal antigenic value. Of the very limited number of strains (four) the examination of which has been described, three (Nos. 21, 22 and 23) were of no greater immunising value than dead vaccines. The fourth strain, '45, guinea pig passage generation 7' (Nos. 19 and 20) conferred a consistently good degree of immunity on the two occasions upon which it was tested. It may be mentioned here that the American strains '805' and '85X' (Nos. 22 and 23) so long regarded with favour by Dr Huddleson have now been admitted to be of little value in producing a serviceable degree of immunity in cattle (Huddleson 1939B).

One serious objection, however, may be raised to the use of living avirulent culture in adult cattle. In the literature available to the author it has never been shown that an avirulent strain of Br. abortus, when passaged in series through its natural host, will not revert to a virulent CO_2 sensitive strain. It would appear therefore, that until it can be shown satisfactorily that a vaccine strain is incapable of such a reversion on passage, it would be advisable to confine its use to young animals only, the so-called 'calfhood vaccination', originally suggested by Buck (1930). He and his co-workers (Cotton, Buck and Smith 1934; Buck, Cotton and Smith 1938) have had considerable success in the immunisation of cattle by this method as had Thomsen (1939) in Denmark. Vaccination is performed when the animals are between four and eight months of age, using for preference a strain of Brucella of relatively /

relatively low virulence and immunity has been shown to persist over more than one pregnancy. The method has several obvious advantages, not the least of which is the greatly decreased tendency for the organism to localise in the udder.

There can be little doubt, that calfhood vaccination is the method of choice in the control of contagious abortion. It cannot of course be regarded as the ideal; the disease will never be eradicated by its use, but 'it is safe to recommend vaccination of calves as a valuable temporary means to reduce the number of abortions' (Thomsen 1939).

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SUMMARY.

1. The work described in this thesis had as its main object an investigation of the possibilities of the immunisation of guinea pigs against infection by Br. abortus.

2. Preliminary work consisted of the development of a method for the expression of the degree of infection of groups of guinea pigs with Br. abortus as a numerical figure; and experiments on pathogenesis.

The degree of infection of each individual guinea pig was estimated by plating dilutions, in multiples of ten, of body tissues on blood agar. The highest dilution in which Brucella was found was taken to be the degree of infection in that tissue, and was represented by the index figure of the dilution. The average of these figures for a group of guinea pigs was accepted as the degree of infection in that particular group.

The pathogenesis of three strains of Br. abortus was undertaken and is described in some detail. The infection caused by the inoculation of a minimal infecting dose of a virulent strain reached its peak about six weeks later, when it gradually declined, whereas two avirulent strains, inoculated in massive doses, were unable to establish themselves in the guinea pig and were eliminated in a few weeks.

3. The efficiency of fifteen dead vaccines, prepared in a variety of ways, and living vaccines consisting of four avirulent strains and one virulent strain of Br. abortus in protecting guinea pigs against infection was determined. The dead vaccines were incapable of affording protection. Of the living cultures, the virulent vaccine conferred an almost complete immunity, one of the avirulent strains produced a consistently good degree of immunity while the remaining three strains were of no greater value than dead vaccine.

4. The significance of these findings is discussed with particular reference to the immunisation of cattle against contagious abortion.

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O. 38

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